Hyperoxia-induced changes in antioxidant capacity and the effect of dietary antioxidants

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[1]*Jean Mayer Human Nutrition Research Center on Aging at Tufts University, Agriculture Research Service, United States Department of Agriculture, Boston, Massachusetts 02111;* [2]*Nutritional Science Department, University of Connecticut, Storrs, Connecticut 06269; and* [3]*Department of Veterans Affairs Medical Center, University of Colorado Health Sciences Center, Denver, Colorado 80262*

Cao, Guohua, Barbara Shukitt-Hale, Paula C. Bickford, James A. Joseph, John McEwen, and Ronald L. Prior. Hyperoxia-induced changes in antioxidant capacity and the effect of dietary antioxidants. *J. Appl. Physiol.* 86(6): 1817–1822, 1999.—We investigated, by measuring oxygen radical absorbance capacity (ORAC), whether hyperoxia causes alterations in antioxidant status and whether these alterations could be modulated by dietary antioxidants. Rats were fed for 8 wk a control diet or a control diet supplemented with vitamin E (500 IU/kg) or with aqueous extracts (ORAC: 1.36 mmol Trolox equivalents/kg) from blueberries or spinach and then were exposed to air or >99% O_2 for 48 h. Although the constituents of the extracts were not extensively characterized, HPLC indicated that blueberry extract was particularly rich in anthocyanins, and the spinach extract did not contain any anthocyanins. The ORAC was determined in samples without proteins [serum treated with perchloric acid (PCA); $ORAC_{PCA}$] and with proteins ($ORAC_{tot}$). Hyperoxia induced a decrease in serum protein concentration, an increase in serum $ORAC_{PCA}$, decreases in lung $ORAC_{PCA}$ and $ORAC_{tot}$, and an equilibration of proteins and $ORAC_{PCA}$ between serum and pleural effusion. These alterations suggested a redistribution of antioxidants between tissues and an increase in capillary permeability during hyperoxia. Only the blueberry extract was effective in alleviating the hyperoxia-induced redistribution of antioxidants between tissues.

oxygen radical absorbance capacity; α-tocopherol; spinach; blueberry

HYPEROXIA IS THOUGHT TO INCREASE the production of reactive oxygen species (ROS) and disrupt the antioxidant defense mechanisms (23). Under basal conditions, ROS are generated during various cellular processes, but are counteracted by well-integrated antioxidant systems, which include enzymes such as superoxide dismutase, catalase, and glutathione peroxidase; macromolecules such as albumin, ceruloplasmin, and ferritin; and an array of low-molecular-weight (LMW) antioxidants, including ascorbic acid, α-tocopherol (vitamin E), β-carotene, reduced glutathione (GSH), uric acid, and bilirubin.

Previous studies showed that, with hyperoxia, oxygen radical production increased in rat lung slices, mitochondria (15), and homogenates (16) and in sheep pulmonary endothelial cells (43). An increased lipid peroxidation was reported in rat brain, lung, and kidney during hyperoxia by measuring fluorescent chromolipids (1). In general, the exposure of animals to hyperoxia results in a significant increase in pulmonary levels of superoxide dismutase, catalase, and glutathione peroxidase (12, 22, 27, 37), although a recent report does not support this conclusion (5). However, in contrast to the large number of studies related to the adaptive responses of antioxidant enzymes to hyperoxia, studies on the effects of hyperoxia on nonenzymatic individual antioxidants are not as prevalent. Additionally, the responses to hyperoxia of these nonenzymatic antioxidants are somewhat inconsistent and not well explained, and the effect of hyperoxia on the total nonenzymatic antioxidant capacity has not been investigated. With hyperoxia, ascorbate decreased and dehydroascorbate increased in lung, whereas both ascorbate and dehydroascorbate increased in plasma (35); lung GSH decreased in old rats (5) but increased in neonatal and young rats (5, 26); and lung oxidized glutathione disulfide (GSSG) and the GSSG/GSH ratio increased in one study (40) and yet remained unchanged in another (26). Hyperoxia did not influence plasma, brain, and lung vitamin E status in guinea pigs (25).

The nonenzymatic antioxidants, most of which have low molecular weights and are able to directly and efficiently quench free radicals, constitute an important aspect of the body's antioxidant mechanism. The limited and inconsistent reports about the response of some individual nonenzymatic antioxidants, mainly ascorbic acid and glutathione, to hyperoxia are not surprising, since the nonenzymatic antioxidant system includes many components, and there are potential interactions among these components. This makes the measurement of individual antioxidants difficult and also less informative while the measurement of total antioxidant capacity becomes necessary and more important in many conditions. Our oxygen radical absorbance capacity (ORAC) assay (6, 11) is one of the tests recently developed to measure the total antioxidant capacities of biological samples. The main advantage of the ORAC assay over other similar methods is its application of the area-under-curve technique in the quantitation process, giving consideration to both inhibition time and inhibition percentage of free radical action by an analyzed antioxidant sample (7, 8, 11). The

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ORAC assay has been used by different laboratories and has provided significant information regarding the antioxidant capacity of various biological samples, from pure compounds such as melatonin, dopamine, and flavonoids to complex matrices such as tea, fruits, vegetables, herbs, and animal tissues (7, 8). By using the ORAC assay in this rat study, we found that hyperoxia induced significant alterations in the total antioxidant capacity in serum and lung, suggesting a redistribution of antioxidants between tissues during hyperoxia. Dietary supplementation of a blueberry extract, which is rich in antioxidant anthocyanins, modulated these hyperoxia-induced alterations in the total antioxidant capacity.

MATERIALS AND METHODS

Reagents. R-phycoerythrin (lot 10H40582) from *Porphyra tenera* ("Nori") was purchased from Sigma Chemical (St. Louis, MO). The R-phycoerythrin lost >90% of its fluorescence within 30 min in the presence of 4 mM of 2,2'-azobis(2-amidinopropane) dihydrochloride, obtained from Waco Chemicals (Richmond, VA). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was obtained from Aldrich Chemical (Milwaukee, WI).

Animals and diets. The use of animals was conducted in compliance with all applicable laws and regulations as well as the principles expressed in the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals.* Forty-eight male Fischer 344 rats (6 mo old) were purchased from Harlan Sprague-Dawley/NIA (Indianapolis, IN). After their arrival, rats were transferred to a barrier facility at the University of Colorado Health Science Center in Denver, CO. All rats were acclimatized for the first 2 wk and then given a control diet or a control diet supplemented with either 500 IU/kg vitamin E acetate, 0.85% spinach extract, or 2.5% blueberry extract. The rats were divided randomly into 4 groups of 12 rats each: Control, Vitamin E, Spinach, and Blueberry. Both spinach and blueberries have a high antioxidant capacity, as assessed by the ORAC assay (9, 32). The blueberry extract was particularly rich in antioxidant anthocyanins (1.143 g/kg or ~42% of the total phenolics), whereas the spinach extract did not contain any anthocyanins (Fig. 1). The amounts of spinach and blueberry extracts added into the control diet were based on the ORAC values of the extracts; they provided an equal ORAC activity (1.36 mmol Trolox equivalent/kg diet). These extracts were prepared by blending fresh spinach or frozen blueberries with deionized water (1:1, wt/vol), centrifuging the homogenates (13,000 *g* at 4°C for 15 min), and lyophilizing the supernatants. Based on the extract weight, blueberries had a higher yield than spinach. The diets were formulated by Research Diets (New Brunswick, NJ), and the composition of the control diet is presented in Table 1. The amount of cornstarch in the control diet was adjusted accordingly when vitamin E acetate, spinach, or blueberry extract was added. The rats were fed these experimental diets for 8 wk before they were exposed to either air or hyperoxia.

Normobaric hyperoxic exposure. One-half of the animals in each group were given hyperoxic exposure for 48 h. The chambers were Plexiglas cylinders (90 cm long × 45 cm in diameter), which held two standard rat cages. Oxygen concentrations in excess of 99% were maintained by flow of 100% oxygen through the chamber at 12–15 l/min. The chambers were pressurized to 760 mmHg by regulating resistance to air outflow. Carbon dioxide concentrations were maintained below 0.5% by addition of soda lime in the bottom of the chamber; temperatures remained between 23 and 25°C, and humidity was maintained at 35–65%. Water and food were available at all times. Rat housing density in the chambers was such that additional food and water, or clean bedding, were not needed.

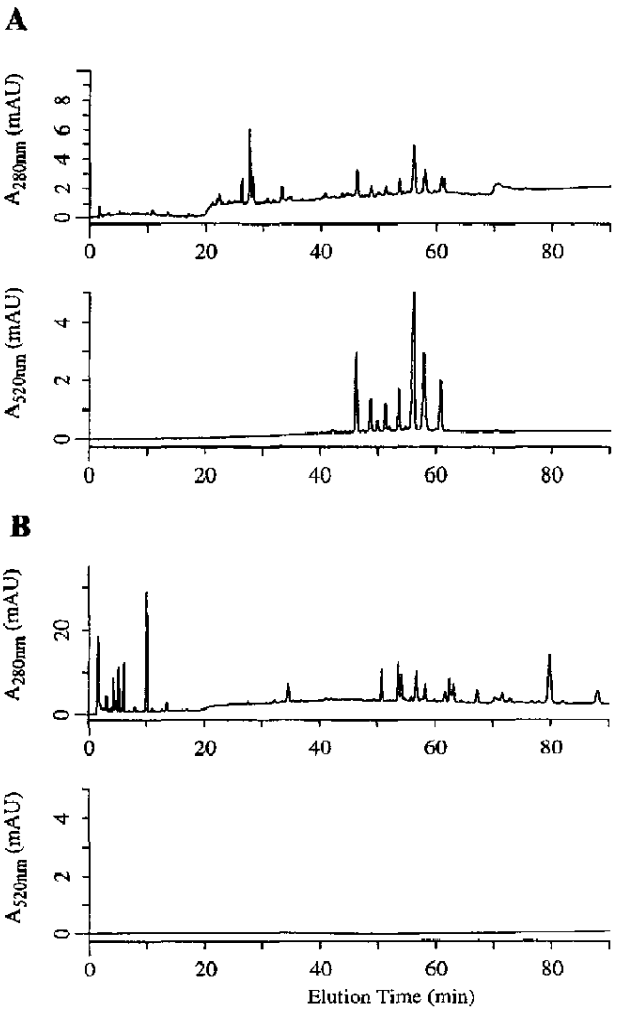

Fig. 1. HPLC chromatograms of a blueberry extract (*A*; 1.0 g/l) and spinach extract (*B*; 0.5 g/l). HPLC system included an HP binary pump, a Zorbax SB-C_{18} column (4.6 × 250 nm), and an HP diode array detector. Absorption (A) of column effluent was recorded at 280 and 520 nm (A_{280nm} and A_{520nm}, respectively). A binary linear gradient method was used as follows: 0–60 min, *mobile phase B* 0–35%; 60–90 min, *mobile phase B* 35–38%. *Mobile phase A*: 25 mM sodium acetate in water. *Mobile phase B*: 25 mM sodium acetate in methanol. Both mobile phases were adjusted to pH 1.5 with trifluoroacetic acid. Flow rate was maintained at 1.0 ml/min. Sample injection volume: 40 µl. AU, arbitrary units.

ORAC assay. Rats were killed by decapitation within 30 min after exposure to air or hyperoxia. Serum and lung were collected as was the pleural effusion from rats exposed to hyperoxia. Serum was diluted 150-fold with 75 mM phosphate buffer (pH 7.0) before it was used in the ORAC ($ORAC_{tot}$) assay. For preparation of serum nonprotein fraction, the serum was diluted with 0.5 M perchloric acid (PCA;

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Table 1. *Composition of control diet*

Ingredient	Composition, g/kg
Casein	189.6
L-Cystine	2.8
Cornstarch	450.2
Maltodextrin 10	118.5
Sucrose	94.8
Cellulose, BW200	47.4
Soybean oil	42.7
Salt mix	9.5
$CaHPO_4$	12.3
$CaCO_3$	5.2
Potassium citrate	15.6
Sodium selenite	0.00009
V13401 vitamin mix[2]	9.5
Choline bitartrate	1.9

Salt mix contains (in g/kg salt mix): 259 NaCl, 41.9 MgO, 257.6 $MgSO \cdot 7H_2O$, 1.925 $CrKSO_4 \cdot 12H_2O$, 1.05 $CuCO_4$, 0.035 KI, 21.0 $FeC_6H_5O_7$, 12.25 $MnCO_4$, 5.6 $ZnCO_4$, and 399.64 sucrose. Vitamin mix contains (in g/kg vitamin mix): 0.8 vitamin A palmitate (500,000 IU/g), 1.0 vitamin D_3 (100,000 IU/g), 0.08 menadione sodium bisulfate (62.5% menadione), 2.0 biotin (1.0%), 1.0 cyanocobalamin (0.1%), 0.2 folic acid, 3.0 nicotinic acid, 1.6 calcium pantothenate, 0.7 pyridoxine·HCl, 0.6 riboflavin, 0.6 thiamin·HCl, and 988.42 sucrose.

1:1, vol/vol) and centrifuged at 4°C for 10 min. The supernatant was recovered for the ORAC ($ORAC_{PCA}$) assay. Pleural effusion was measured for its volume and centrifuged at 1,600 *g* for 10 min (4°C). The supernatant was recovered and then processed for the ORAC assay as the serum samples. Lung tissues were homogenized by using the phosphate buffer (1:4 wt/vol). Cytosol was separated by a two-step centrifugation process (12,000 *g* for 10 min followed by 100,000 *g* for 15 min at 4°C). The cytosol samples were then diluted with buffer for the $ORAC_{tot}$ assay and treated further with 0.5 M PCA for the $ORAC_{PCA}$ assay.

The automated ORAC assay was carried out on a COBAS FARA II spectrofluorometric analyzer (Roche Diagnostic System, Branchburg, NJ) (7, 11).

Statistics. Data are means ± SE. The effects of diet and hyperoxia, as well as their interaction, on the protein contents and the antioxidant capacities measured as $ORAC_{tot}$ and $ORAC_{PCA}$ in serum, pleural effusion, and lung were analyzed by two-way ANOVA by using Systat software (Systat, Evanston, IL). Multiple pairwise comparisons were evaluated by Tukey's honestly significant difference test using Systat software. Differences at $P < 0.05$ were considered significant.

RESULTS

The HPLC chromatograms shown in Fig. 1 for the blueberry and spinach extracts indicate that the blueberry extract is particularly rich in anthocyanins, whereas spinach extract does not contain any anthocyanins. Anthocyanins are characterized by two absorption peaks, which are at 280 and 520 nm, respectively. Other flavonoids have absorption peaks at 280 nm but not at 520 nm.

The weights of rats in each group increased by 6–8% after the feeding of the diets for 8 wk, with no significant differences observed between different diet groups.

Effects of diets and hyperoxia on serum protein concentration and antioxidant capacity. The effects of diets and hyperoxia on serum protein and ORAC are shown in Table 2. Serum protein concentrations decreased significantly with hyperoxia in all animals except those receiving blueberry extract. Compared with the air-exposed controls, serum $ORAC_{tot}$ increased significantly in the air-exposed rats receiving blueberry or spinach extract but not in the air-exposed rats receiving vitamin E. Hyperoxia had no significant effect on the serum $ORAC_{tot}$ in any group, but it significantly increased serum $ORAC_{PCA}$ in all animals except those receiving blueberry extract. Serum $ORAC_{PCA}$ was not significantly affected by hyperoxia in the animals receiving blueberry extract.

Protein concentration, antioxidant capacity, and volume of the pleural effusion from the rats exposed to hyperoxia. The rats exposed to >99% oxygen for 48 h showed obvious lung edema and pleural effusion, two typical pathological changes seen in lung oxygen toxicity. There was no lung edema, and visible pleural effusion formed in the rats exposed to air for 48 h. As

Table 2. *Protein concentration and ORAC in the serum and pleural effusion of rats exposed to air or >99% O_2 for 48 h*

	Serum			Pleural Effusion		
Group	Protein, mg/ml	$ORAC_{tot}$, mM Trolox equivalents	$ORAC_{PCA}$, μM Trolox equivalents	Protein, mg/ml	$ORAC_{tot}$, mM Trolox equivalents	$ORAC_{PCA}$, μM Trolox equivalents
Control						
Air	114.0 ± 5.2	4.34 ± 0.24	576 ± 46			
O_2	93.4 ± 3.6*	4.55 ± 0.17	736 ± 58*	94.2 ± 4.9	3.13 ± 0.49	739 ± 32
Vitamin E						
Air	111.4 ± 3.8	4.51 ± 0.13	634 ± 53			
O_2	96.5 ± 1.2*	4.94 ± 0.18	825 ± 45*	98.4 ± 4.9	3.50 ± 0.43	746 ± 50
Spinach						
Air	116.5 ± 3.7	5.01 ± 0.12†	574 ± 38			
O_2	89.7 ± 4.3*	4.89 ± 0.29	744 ± 56*	87.2 ± 3.7	2.82 ± 0.19	771 ± 29
Blueberry						
Air	114.7 ± 4.6	4.98 ± 0.11†	625 ± 50			
O_2	106.8 ± 3.0	5.46 ± 0.41	634 ± 75	88.3 ± 2.7‡	2.95 ± 0.13	844 ± 31‡

Values are means ± SE. $ORAC_{tot}$, oxygen radical absorbance capacity of untreated serum; $ORAC_{PCA}$, oxygen radical absorbance capacity of the serum nonprotein fraction treated with perchloric acid (PCA). There was no visible pleural effusion in rats exposed to air. See text for further description. *O_2 vs. air, $P < 0.05$; †Spinach or Blueberry vs. Control group, $P < 0.05$; ‡pleural effusion vs. serum, $P < 0.05$.

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shown in Table 2, the protein concentrations and the $ORAC_{PCA}$ values of the pleural effusion were not significantly different from those of serum in the rats exposed to hyperoxia in the Control, Vitamin E, and Spinach groups. However, the protein concentration was significantly lower, and the $ORAC_{PCA}$ value was significantly higher, in the pleural effusion compared with the serum in the rats receiving blueberry extract. The pleural effusion $ORAC_{tot}$ was significantly lower than serum $ORAC_{tot}$ in all the rats exposed to hyperoxia. However, the volume of pleural effusion formed in the rats exposed to hyperoxia was not significantly different between the Control (3.18 ± 0.16 ml) and Vitamin E (2.98 ± 0.38 ml), Spinach (3.40 ± 0.34 ml), or Blueberry (3.40 ± 0.22 ml) group.

Effects of diets and hyperoxia on lung antioxidant capacity. The effects of diets and hyperoxia on lung cytosol ORAC are shown in Table 3. Lung cytosolic $ORAC_{tot}$ decreased significantly with hyperoxia in all groups. Lung cytosolic $ORAC_{PCA}$ decreased significantly with hyperoxia in Control, Vitamin E, and Spinach groups (ANOVA, effect of oxygen, $P < 0.05$) but not in the Blueberry group.

DISCUSSION

The free radical theory of oxygen toxicity is generally accepted. According to this theory, oxygen toxicity is a result of the overproduction of ROS. The primary site of injury in normobaric oxygen toxicity is the lung. One of the characteristics of oxygen toxicity is the increased capillary permeability resulting in lung edema and pleural effusion. However, when the free radical theory was tested in vivo, nonenzymatic parameters were usually examined in lung and serum or plasma but not the pleural effusion (1, 5, 25, 26, 35, 40). When such parameters were examined, only limited individual antioxidants were considered (1, 5, 25, 26, 35, 40).

In the present study, the total antioxidant capacity in serum, lung, as well as pleural effusion was investigated by using the ORAC assay. Our results showed that hyperoxia caused *1*) a decrease of protein concentration, an increase of $ORAC_{PCA}$, and no change of $ORAC_{tot}$ in serum; *2*) a decrease of both $ORAC_{PCA}$ and $ORAC_{tot}$ in lung; and *3*) the formation of pleural effusion, which had the same protein concentration and $ORAC_{PCA}$ as serum did. Although the parameters measured in this study were obtained under static conditions, we have developed a dynamic model to help understand some of the changes observed. These results can be explained by using the model depicted in Fig. 2. The opposing movements of albumin, a macromolecular antioxidant, and LMW antioxidants between the bloodstream and lungs during hyperoxia result in the unchanged $ORAC_{tot}$ in serum, which measures both albumin and LMW antioxidants. Under normal conditions, the LMW antioxidant concentration in lung cytosol is much higher than that in serum, as demonstrated in this study using the $ORAC_{PCA}$ assay, which measures the total antioxidant capacity from nonprotein components extracted with PCA. The $ORAC_{PCA}$ in lung cytosol (3.2 ± 0.2 mM Trolox equivalents, $n = 22$) was more than fivefold that in serum (0.6 ± 0.02 mM Trolox equivalents, $n = 22$) in the air-exposed rats. The movement of LMW antioxidants from the lung into the bloodstream during hyperoxia caused the increase of serum $ORAC_{PCA}$ and the decrease of lung $ORAC_{PCA}$. The hyperoxia-induced capillary-alveolar albumin leak has been clearly demonstrated in other studies (13, 41).

Increased consumption of fruits and vegetables has been associated with protection against various diseases (18, 34, 36). It is not known what active dietary constituents contribute to these protective effects, but it is often assumed that antioxidant nutrients contribute to this defense (3, 4, 21, 42). By using ORAC assay, we found that blueberries and spinach had high antioxidant capacities (9, 32), which were 20–50 times higher that those of some other fruits and vegetables, such as honeydew melon and cucumber, on a fresh-weight basis. The results of the present study suggested that the hyperoxia-induced redistribution of proteins and LMW antioxidants between bloodstream, lung, and pleural effusion was blocked, at least in part, by dietary supplementation of blueberry extract for 8 wk. The blueberry extract significantly increased se-

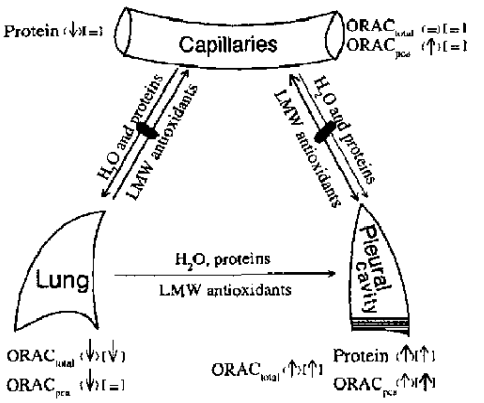

Fig. 2. Model used to explain hyperoxia-induced alterations in antioxidant capacity in serum, lung, and pleural effusion, which were blocked by dietary supplementation of blueberry extract. This model is based on data from this animal study. $ORAC_{tot}$, oxygen radical absorbance capacity of untreated serum; $ORAC_{PCA}$, ORAC of the serum nonprotein fraction treated with perchloric acid (PCA); LMW, low-molecular-weight; ↑, increase; ↓, decrease; =, no change; thick black mark, inhibition site by blueberry extract; ( ), hyperoxia; [ ], hyperoxia with dietary blueberry extract supplementation.

Table 3. *ORAC in the lung cytosol of rats exposed to air or >99% O_2 for 48 h*

Group	$ORAC_{tot}$		$ORAC_{PCA}$	
	Air	O_2	Air	O_2
Control	96.1 ± 8.5	70.8 ± 3.7*	31.3 ± 6.6	18.7 ± 3.4†
Vitamin E	96.2 ± 11.0	75.2 ± 5.9*	26.0 ± 2.1	21.0 ± 4.8†
Spinach	99.3 ± 5.2	76.1 ± 4.1*	24.8 ± 3.2	14.9 ± 2.0†
Blueberry	102.4 ± 5.5	74.6 ± 6.7*	26.5 ± 2.7	24.6 ± 5.2

Values are means ± SE. $ORAC_{tot}$ and $ORAC_{PCA}$ values expressed in nmol Trolox equivalent/mg protein. *O_2 vs. air (post hoc Tukey's test), $P < 0.05$; †O_2 vs. air (ANOVA or pooled *t*-test without Blueberry group), $P = 0.016$.

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rum $ORAC_{tot}$ and it prevented the hyperoxia-induced *1*) decrease of serum protein, *2*) increase of serum $ORAC_{PCA}$, and *3*) decrease of lung $ORAC_{PCA}$. The protein concentration was significantly higher and the $ORAC_{PCA}$ was significantly lower in the serum than in pleural effusion in the rats receiving blueberry extract and exposed to hyperoxia. These observed effects might be attributed to the abundant antioxidant components in the blueberry extract. However, the antioxidant components responsible for the effects of blueberry extract must be different from the components in spinach; an equal amount of antioxidant activity from spinach was ineffective in preventing the redistribution of proteins and LMW antioxidants, although the spinach treatment also significantly increased serum $ORAC_{tot}$ (Table 2). Therefore, the blueberry components responsible for the observed effects in this study could be antioxidants or non-antioxidants. Although the plant extracts used in this study were not extensively chemically characterized, HPLC was used to show that the blueberry extract is rich in anthocyanins, as reported by us and others (1.28 mg/g fresh wt or 9.11 mg/g dry wt) (24, 32), whereas spinach does not contain any anthocyanins (Fig. 1). Anthocyanins are easily absorbed in rats (30). Anthocyanins protected against the increase of capillary permeability induced in *1*) rabbits by topical application of chloroform and intradermal injection of bradykinin; *2*) rats by hypertension; and *3*) hamsters by diabetes, ischemia, and reperfusion (2, 14, 17, 20, 28, 29, 31). One of the mechanisms underlying these protective effects was thought to be the effects of anthocyanins on the proteolytic enzymes. Anthocyanins inhibit proteolytic enzymes like elastase, which are involved in the degradation of collagen and other components of the extravascular matrix in certain pathological conditions (29). The antioxidant properties of anthocyanins may contribute to these protective effects but may not be the only or primary reason. Therefore, anthocyanins or other, as-yet-unidentified, components of the blueberry extract may be responsible for the observed protection against the hyperoxia-induced increase in capillary permeability.

In summary, hyperoxia caused significant changes in antioxidant capacity in rat serum and lung. These changes were modulated by the dietary supplementation of an aqueous extract from blueberries, but not by vitamin E or an aqueous extract from spinach.

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